Insulin protects islets from apoptosis via Pdx1 and specific changes in the human islet proteome

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Insulin is both a hormone regulating energy metabolism and a growth factor. We and others have shown that physiological doses of insulin initiate complex signals in primary human and mouse β-cells, but the functional significance of insulin's effects on this cell type remains unclear. In the present study, the role of insulin in β-cell apoptosis was examined. Exogenous insulin completely prevented apoptosis induced by serum withdrawal when given at picomolar or low nanomolar concentrations but not at higher concentrations, indicating that physiological concentrations of insulin are antiapoptotic and that insulin signaling is self-limiting in islets. Insulin treatment was associated with the nuclear localization of Pdx1 and the prosurvival effects of insulin were largely absent in islets 50% deficient in Pdx1, providing direct evidence that Pdx1 is a signaling target of insulin. Physiological levels of insulin did not increase Akt phosphorylation, and the protective effects of insulin were only partially altered in islets lacking 80% of normal Akt activity, suggesting the presence of additional insulin-regulated antiapoptotic pathways. Proteomic analysis of insulin-treated human islets revealed significant changes in multiple proteins. Bridge-1, a Pdx1-binding partner and regulator of β-cell survival, was increased significantly at low insulin doses. Together, these data suggest that insulin can act as a master regulator of islet survival by regulating Pdx1.

diabetes | pancreatic β-cells | programmed cell death | autocrine insulin feedback signaling | maturity onset diabetes of the young

Interest in the effects of insulin on pancreatic β-cells has increased after the observation that ablation of insulin receptors in pancreatic β-cells (βIRKO) led to a decrease in β-cell mass and age-dependent diabetes (1–3). Similarly, $Irs2^{-/-}$ mice have reduced β-cell mass and increased islet apoptosis (4–6). Genetic evidence suggests that Irs2 and Foxo1, a negative regulator of insulin signaling, may be upstream regulators of the homeobox transcription factor Pdx1 (4–7), a master regulator of the morphogenesis and survival of multiple cell types in the pancreas and gut (8–10). These genetic findings suggest the possibility that Pdx1 may mediate the effects of insulin. Recently, insulin receptor and Irs2 mRNA levels were shown to be decreased in islets isolated from human type 2 diabetics (11). Nevertheless, the significance of insulin signaling in primary β-cells has been questioned, in part because of the failure to demonstrate consistently direct effects of insulin at physiological doses on specific β-cell functions (12). We have shown recently in human β-cells that insulin generates intracellular Ca^{2+} signals and stimulates a transient increase in insulin synthesis but that insulin does not have significant acute effects on its own secretion (13, 14). The goals of the present study were to determine whether insulin altered apoptosis in primary human and mouse islets and to examine the mechanism by which insulin acts on these cells.

The present study demonstrates that insulin has potent antiapoptotic effects in primary human and mouse islets that are mediated through the antiapoptotic transcription factor Pdx1. Furthermore, our results show that the antiapoptotic effects of insulin are associated with specific changes in the human islet proteome. Thus, insulin signaling pathways in the β-cell may be possible therapeutic targets for treating diabetes by promoting the survival of β-cells.

Results

Physiological Doses of Insulin Protect Islets from Apoptosis. The effect of multiple doses of insulin on islet apoptosis was examined by measuring the organized self-disassembly of genomic DNA as reflected by the extent of DNA laddering. DNA ladders are a hallmark of apoptosis but not necrosis; however, they typically require large amounts of starting material. Critically, the islet PCR-enhanced DNA ladder technique (8, 15) made it possible to study small groups of islets, thereby reducing the ambient insulin accumulating in the cultures. Experiments were performed at 5 mM glucose to minimize further the release of endogenous insulin. We confirmed by radioimmunoassays that endogenous insulin accumulation was negligible in our cultures (supporting information (SI) Fig. 6). Under these conditions, low nanomolar concentrations of exogenous insulin (0.2–20 nM) were effective in rescuing both human and mouse islets from death induced by serum withdrawal (Fig. 1 *A–D*). Interestingly, higher concentrations of insulin (200 nM) did not show additional protective effects. We have shown that β-cells respond to insulin with a similar bell-shaped dose–response curve (13, 14). In preliminary studies, 15 mM glucose also protected human islets from serum-free conditions (J.D.J., unpublished data), further suggesting that autocrine/paracrine insulin signaling is antiapoptotic. To determine whether insulin was preventing caspase-3 activation before DNA fragmentation, human islets were examined by Western blot after 3-day treatment with various insulin concentrations. Low doses of insulin, but not high doses, prevented caspase-3 cleavage (Fig. 1 *E* and *F*). Together, these results indicate that low concentrations of insulin promote islet survival, an effect that may be lost at higher concentrations of the hormone.

Role of Pdx1 in Antiapoptotic Insulin Signaling. Potential antiapoptotic downstream targets of insulin signaling were evaluated next. Pdx1 is a transcription factor that is critical for the development and differentiation of the pancreas (16). Pdx1 was considered as a potential candidate to mediate the antiapoptotic effects of insulin because we have recently implicated Pdx1 in the regulation of islet apoptosis *in vitro* and *in vivo* (8). Because the translocation of Pdx1 to the nucleus is a critical event for its activity as a transcription factor, the subcellular location of Pdx1 was examined in cultured β-cells by using immunofluorescence. After 3 days of culture in

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Abbreviation: PI3K, phosphatidylinositol 3-kinase.

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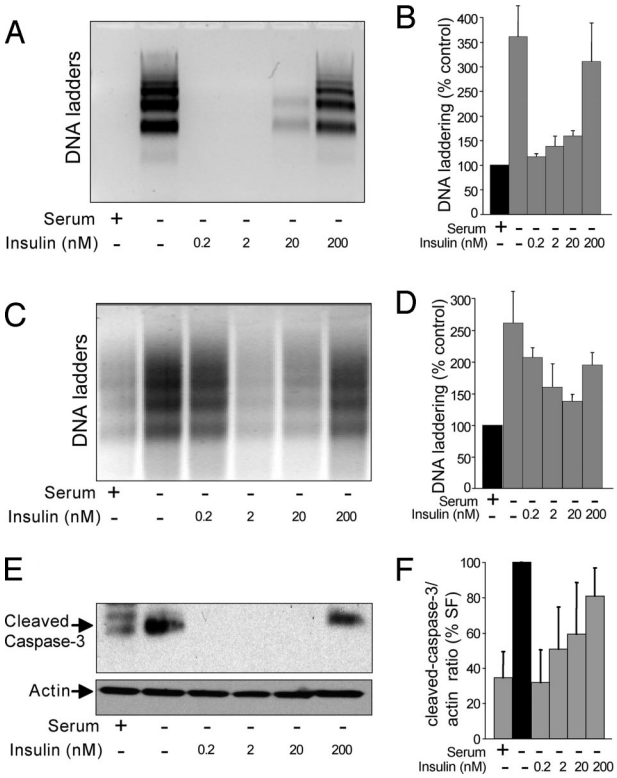

Fig. 1. Insulin is a potent antiapoptotic factor. Insulin protected groups of five mouse islets (*A*; $n = 4$) or five human islets (*C*; $n = 6$) cultured for 7 days in serum-free conditions, as assessed by the PCR-enhanced DNA laddering assay. Results are quantified for each experiment in *B* and *D*. (*E* and *F*) Low doses of insulin selectively reduced cleaved caspase-3 in human islets.

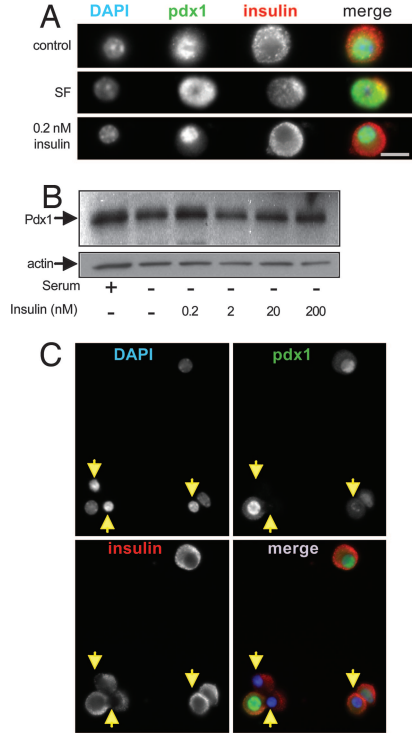

Fig. 2. Insulin stimulates Pdx1 translocation in primary mouse β-cells. (*A*) Dispersed mouse islet cells cultured for 3 days as indicated are shown triple-labeled with DAPI to delineate nuclei, Pdx1 antibody, and insulin antibody. (*B*) Western blot of Pdx1 and β-actin in mouse islets treated as indicated for 3 days. Pooled densitometry data from three independent experiments indicated that Pdx1 protein expression in 0.2 nM insulin was 130 ± 1% ($P < 0.05$) of that in the serum-free control. None of the other treatments were significantly different from control. (*C*) Dispersed mouse islet cells cultured for 3 days in 0.2 nM insulin were labeled as above. Arrows denote apoptotic cells with condensed nuclei and little or no Pdx1 immunoreactivity. Nonapoptotic cells exhibit high levels of Pdx1 in a nuclear pattern. Experiments were repeated independently with similar results by using islets from four separate mice (total $n = >400$ cells for each condition).

control conditions (5 mM glucose, 10% serum), Pdx1 was predominately localized to the nucleus in mouse β-cells (Fig. 2*A*). On the other hand, Pdx1 was located primarily in the cytoplasm in cultures lacking serum. Treatment with insulin strongly stimulated Pdx1 translocation to the nucleus (Fig. 2*A*). Preliminary experiments indicate that this effect can be seen within 6 h of exposure to 0.2 nM insulin (E.U.A. and J.D.J., unpublished data). As in our studies of apoptosis, 200 nM insulin was not more effective than 0.2 nM insulin (SI Fig. 7 *A–C*). In addition, insulin-treated cells exhibited more intense Pdx1 and insulin staining on average compared with untreated controls. Western blot also showed a very modest increase in Pdx1 protein in islets cultured in 0.2 nM insulin for 3 days (Fig. 2*B* and SI Fig. 7*D*), but not for 7 days (data not shown). On a cell-by-cell basis within cultures, single β-cells with a morphological characteristic of apoptotic cells (condensed nuclei) showed significantly reduced Pdx1 nuclear translocation and expression compared with nonapoptotic cells (Fig. 2*C* and SI Fig. 7 *E–H*). These experiments suggested the possibility that insulin was acting through Pdx1 to block islet apoptosis.

If this hypothesis is correct, the antiapoptotic effects of insulin should be reduced in islets from $Pdx1^{+/-}$ mice compared with wild-type littermate controls. As we have previously shown, the rate of apoptosis was enhanced in $Pdx1^{+/-}$ islets under basal conditions *in vitro* (8). Most concentrations of insulin were not at all protective in $Pdx1^{+/-}$ islets (Fig. 3 *A* and *B*), strongly suggesting that the full action of antiapoptotic insulin signaling requires both alleles of Pdx1. The observation that 20 nM insulin was protective suggests the possibility that certain concentrations of insulin can compensate for a lack of one Pdx1 allele. Together these results suggest that Pdx1 is a critical downstream target of insulin signaling in primary islets. Because Pdx1 is thought to control insulin expression in β-cell lines, we addressed the possibility that Pdx1 haploinsufficiency altered apoptosis by reducing endogenous insulin production. We have demonstrated that neither acute nor chronic insulin secretion is reduced in islets isolated from $Pdx1^{+/-}$ mice compared with littermate controls (8). Insulin content from isolated $Pdx1^{+/-}$ islets was actually higher than wild-type islets (Fig. 3*C*), and glucagon content was unchanged (SI Fig. 7*I*). At the single-cell level, no correlation was observed between Pdx1 translocation and insulin staining intensity (SI Fig. 7*J*). Thus, having both alleles of Pdx1 is important for the antiapoptotic actions of insulin but is dispensable for maintaining insulin expression in primary cells.

We also examined the sustained effects of insulin exposure on the abundance, phosphorylation status, and subcellular localization of Foxo1, a major upstream regulator of Pdx1. Although insulin treatment did not result in a robust change in Foxo1 phosphorylation or total protein levels (SI Fig. 7*L*), we did observe a modest decrease in the percentage of cells exhibiting nuclear Foxo1 staining in insulin-treated cultures, relative to serum-free conditions (SI Fig. 7*M*). Together, these results suggest the possibility that translocation of Foxo1 may participate in antiapoptotic insulin signaling in primary β-cells.

Role of AKT in Islet Insulin Signaling. Akt has been suggested to be a critical component of insulin/Igf1 signaling in β-cells, based largely on studies using high concentrations of insulin or Igf1. In the present study, we examined the effects of various concentrations of insulin

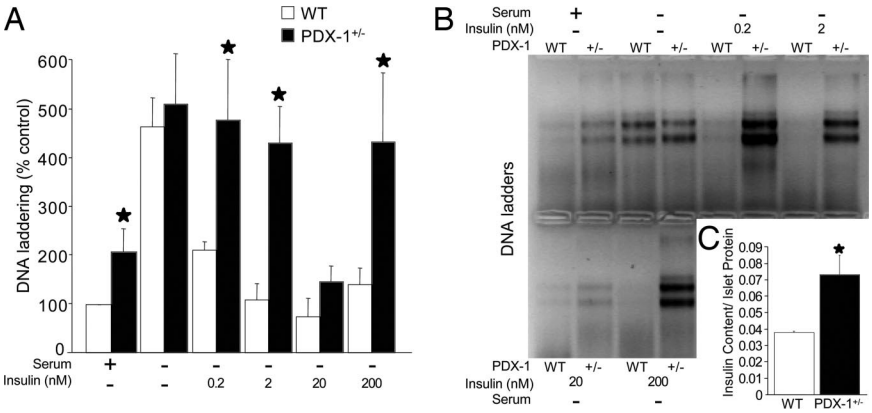

Fig. 3. Pdx1 is required for the antiapoptotic effects of insulin but not insulin protein expression. The protective effects of insulin are largely abrogated in PDX1$^{+/-}$ islets compared with wild-type islets (*A* and *B*). Results are quantified in *B* ($n = 3$). Stars indicate significant difference compared with similarly treated wild-type islets. (*C*) Isolated Pdx1$^{+/-}$ islets have increased insulin protein levels ($n = 3$).

on sustained Akt phosphorylation and total Akt protein levels. Interestingly, compared with serum-free controls, insulin treatment did not result in sustained Akt phosphorylation or a robust increase in Akt protein in mouse islets (Fig. 4*A* and SI Fig. 7*K*) or human islets (data not shown). This suggests that chronic signaling via physiological concentrations of insulin may work through alternative pathways in β-cells. As a positive control for the known acute effects of high concentrations of insulin and Igf1, we also examined Akt phosphorylation after 15 min. In this experiment, both 200 nM insulin and 100 nM Igf1 induced rapid Akt phosphorylation, whereas a concentration of 0.2 nM had no effect on Akt (Fig. 4*B*). This result suggests that low, antiapoptotic doses of insulin may not stimulate Akt in primary β-cells.

Akt plays an antiapoptotic role in many tissues and overexpression of constitutively active Akt increases β-cell mass (17). However, the observations that Akt phosphorylation is associated with prosurvival signaling (18) do not necessarily mean that Akt is a critical component of insulin-dependent antiapoptotic signaling pathways in the β-cell. Indeed, no direct evidence has yet been presented that reducing Akt activity leads to increased apoptosis in primary β-cells. We have shown that transgenic mice expressing a dominant negative, kinase-dead form of Akt under the control of the insulin promoter (Aktdn^{RIP} mice) (19) do not have a decrease in β-cell mass, in contrast to the case of βIRKO and Pdx1$^{+/-}$ mice (2, 8). To determine whether the antiapoptotic effects of insulin required Akt, we measured apoptosis in islets from Aktdn^{RIP} mice treated with different concentrations of insulin. The protective effects of 0.2 nM insulin were blunted in AKTkd^{RIP} islets, suggesting a role for Akt or its downstream targets at this dose of insulin. However, the overall effects of insulin at other concentrations were similar to islets from the wild-type littermate control mice (Fig. 4*C*), indicating that Akt does not play a dominant role in mediating prosurvival signaling at most doses of insulin.

We also examined a proximal event in β-cell insulin signaling, Ca^{2+} mobilization from NAADP- and IP_3-sensitive Ca^{2+} stores. Based on experiments using wortmannin, a phosphatidylinositol 3-kinase (PI3K) inhibitor, it has been suggested that a PI3K/Akt signaling module may be upstream of insulin-induced Ca^{2+} signaling in mouse β-cells (20). However, the Ca^{2+} signals generated by insulin were not blocked in cells isolated from Aktdn^{RIP} mice (Fig. 4*D*).

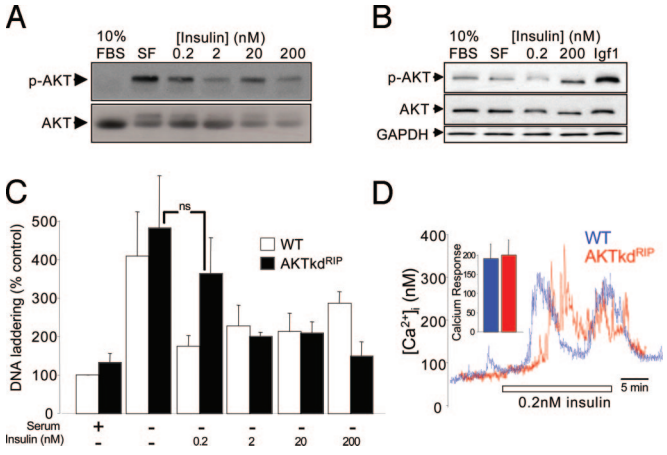

Fig. 4. Role of AKT in anti-apoptotic insulin signaling. (*A*) Akt phosphorylation and total levels in mouse islets cultured for 2 days as indicated. (*B*) Acute (15-min) effects of insulin and 100 nM IGF-1 ($n = 3$). (*C*) Effects of insulin on apoptosis in islets from wild-type and transgenic mice expressing dominant-negative AKT in β-cells ($n = 4–6$). (*D*) Insulin-stimulated Ca^{2+} signals are not altered in single Fura-4F-loaded β-cells from AKTdn^{RIP} mice. Although variability is seen in the shape of insulin-stimulated Ca^{2+} signals, quantification by using mean amplitude, mean area under the curve, or mean rate of rise did not reveal differences. Experiment was replicated on cells from three mice. (*D Inset*) The quantified mean Ca^{2+} signal amplitude.

Effects of Insulin on the Islet Proteome. Differential expression proteomics was used to identify and characterize additional proteins involved in mediating the effects of insulin on β-cell apoptosis. Human islets were used in these experiments because of the requirement for large amounts of protein. To reduce the variability between experiments using 2D gels, a newer method was used whereby multiple samples are imaged on the same gel with fluorescent dyes (SI Fig. 8). This approach allowed the accurate detection and analysis of many more spots, including low-abundance proteins. More than 2,000 individual spots were detected in each human islet gel (Fig. 5*A*). In a typical experiment, the software detected that 4% of spots showed increased intensity, 9% decreased, and 87% were unchanged in the presence of insulin by using 2 standard deviations from the mean as the statistical cutoff to detect significance (Fig. 5*B*). Each spot was manually vetted further to ensure it did not overlap with other proteins. The identities of 36 individual spots significantly changed by insulin are listed in SI Table 1. Several of these spots also were regulated by exposure to high glucose. From this list, it is evident that insulin affects several key pathways in human islet cells, including metabolism, the cytoskeleton, and transcription/translational machinery.

It is important to note that a given gene product can be represented in multiple places on a 2D gel and that only a fraction of the total islet protein species is represented on our lysates/gels. Thus, a "decrease" in a protein spot actually could represent a shift to another location on the gel because of a posttranslational modification or the translocation of the protein to a cellular compartment that is underrepresented in our lysates. As such, these proteomic analyses cannot be used to determine whether the total level of a specific protein increases or decreases, only that it changes. Therefore, it is critical to examine further the proteins of interest by

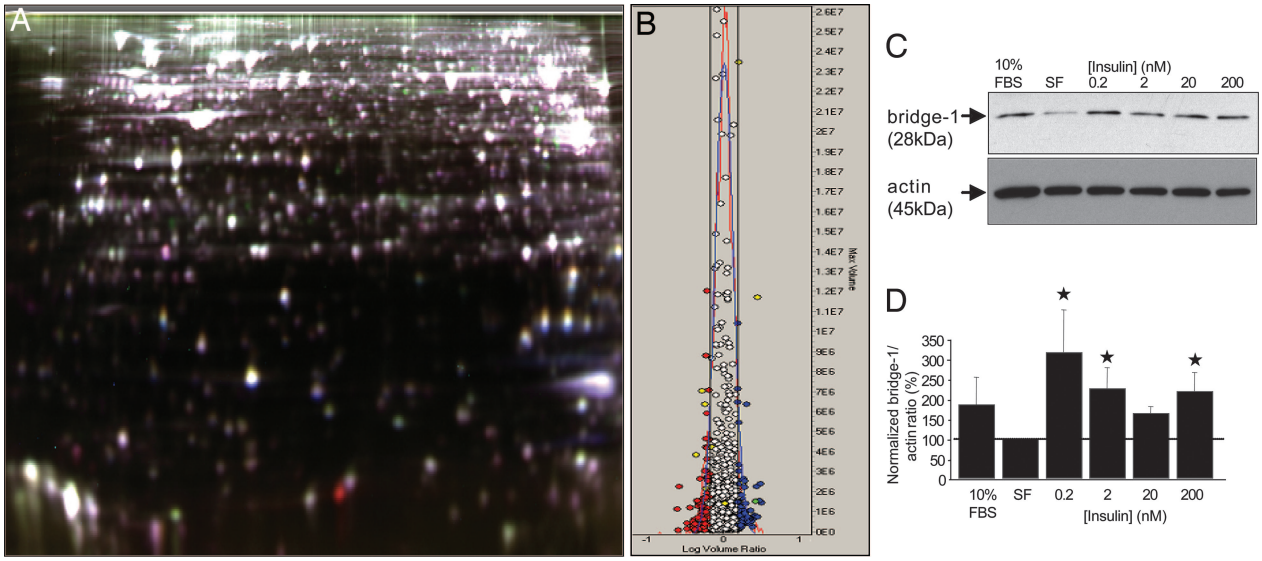

Fig. 5. Fluorescent 2D gel proteomic analysis of human islets treated with insulin. (*A*) Samples of human islets cultured for 36 h with serum-free RPMI medium 1640 containing 5 mM glucose, media containing 5 mM glucose plus 2 nM insulin, or media containing 25 mM glucose were labeled with the fluorescent dyes (Cy2, Cy3, and Cy5, respectively) and run on 15% 2D gels (molecular mass is vertical) with an isoelectric focus range of pH 3–10 (horizontal). Results shown are representative of gels from four separate donors. (*B*) Spots that are significantly changed (>2 SD from the mean; blue, increased with insulin; red, decreased with insulin) are detected by using software. Distribution of log volume ratios (between Cy2 and Cy5) and spot volume (size of spot) are shown. (*C*) Western blot analysis of bridge-1 expression in mouse islets cultured for 3 days as indicated. (*D*) Pooled densitometry results from four independent experiments are quantified. Stars indicate significant difference from serum-free control.

using more conventional techniques such as Western blot and immunofluorescent localization, when antibodies are available.

The observed change in bridge-1/psmd9 (SI Figs. 8 and 9) was particularly interesting because this protein was shown recently to directly interact with Pdx1 *in vitro*, regulate Pdx1 transcriptional activity, and influence β-cell survival *in vivo* (21). Western blot analysis showed that total bridge-1 protein was increased most significantly by low concentrations of insulin (Fig. 5 *C* and *D*). Insulin did not alter the cytosolic localization of bridge-1, unlike Pdx1, or enhance its binding to Pdx1 (SI Fig. 10). Together, our results indicate that insulin increases the expression of bridge-1, a positive regulator of Pdx1.

Western blots were used to confirm that treating human islets with insulin increased the total protein levels of peroxiredoxin V and ATP synthase subunit D and decreased the expression of ARPC2, 14-3-3 ζ/δ and cofilin to varying degrees in at least one dose of the hormone (SI Fig. 10). In some instances, we were unable to demonstrate changes in total protein, suggesting the possibility that posttranslational modifications or movement between cellular compartments may have led to the alterations seen in the 2D gel proteomics.

Discussion

The present studies were undertaken to determine the effects of insulin on the survival of primary islets and resulted in three major findings. First, we have demonstrated that physiological concentrations of insulin protect human and mouse islets from apoptosis that occurs after serum withdrawal and that this system appears to be self-limiting *in vitro*. Second, the antiapoptotic effects of insulin require Pdx1 but can be observed in islets lacking 80% of the normal Akt activity. Third, insulin induced significant changes in the human islet proteome and increased the expression of bridge-1, as well as proteins involved in signaling, the cytoskeleton, β-cell metabolic activity, and transcription/translation.

Although a mounting body of literature has implicated downstream components of growth factor signaling (Irs2, PI3K, Akt, etc.) in islet survival, it remains controversial whether insulin, Igf1, or other growth factors are the major factors driving these pathways. Whereas βIRKO mice have significantly lower β-cell mass (2), β-cell-specific knockout of the Igf1 receptor does not alter β-cell mass or islet apoptosis (3, 22, 23). Recently, it has been reported that MIN6 cells with RNAi knockdown of the insulin receptor are more susceptible to apoptosis induced by ER stress (24). Although previous studies have suggested that superphysiological concentrations of insulin can protect islets from apoptosis (25, 26), those studies used levels of insulin >1,000 times higher than what is found in the portal circulation (27). Because high levels of insulin would be expected to activate Igf1 receptors, many have remained skeptical that insulin itself has antiapoptotic effects in the islet. It is unlikely that the antiapoptotic effects of picomolar concentrations of insulin seen in our study are mediated by Igf1 receptors, which exhibit 500-fold reduced affinity for insulin (28). In addition to providing a description of the antiapoptotic effects of picomolar insulin concentrations, we have identified previously undefined mechanisms by which insulin inhibits apoptosis in the present study. Our data also indicated that insulin signaling, although extremely potent, is inherently self-limiting, with higher doses showing no increase or even reduced efficacy. The atypical dose–response profile may result from several factors including negative cooperativity or desensitization of the insulin receptor (28) or the desensitizing properties of NAADP, a CD38-derived second messenger implicated in β-cell insulin signaling (14, 29).

In the present study, we have elucidated critical components of the antiapoptotic insulin signal transduction cascade. Here, we directly link the ability of insulin to protect β-cells with Pdx1, a master regulator of β-cell survival and function. We and others have shown that Pdx1 is involved in maintaining adequate expression of antiapoptotic proteins, BCl-2 and BCl_{XL} and that loss of Pdx1 leads to increased caspase-3 activity (8, 30). As such, islets from $Pdx1^{+/-}$ mice displayed increased islet apoptosis *in vivo* and *in vitro* before any detectable effect on insulin secretion from isolated islets, as well as a progressive loss in β-cell mass (8). In addition to genetic evidence supporting a link between insulin/Igf1 signaling components and Pdx1 (4–7, 31), our conclusion that Pdx1 is a critical component of insulin signaling is supported by the finding that insulin stimulates DNA-binding activity of Pdx1 in human islets

(32). Notably, this scenario provides an alternate, and more direct, explanation for the observation that glucose homeostasis and β-cell mass are increasingly impaired when Pdx1 heterozygote mice are crossed with mice lacking one allele of the insulin receptor and/or insulin receptor substrate-1 (33). Our data suggest that Pdx1 translocation to the nucleus is a key event in antiapoptotic insulin signaling. Similarly, a previous study in MIN6 cells found that Pdx1 translocation could be stimulated by glucose or 20 nM insulin (34). Interestingly, 0.2 nM insulin stimulated a modest, transient increase in Pdx1 protein expression, evident after 3 days but not 7 days. Similarly, transient RNAi knockdown of insulin receptors reduced Pdx1 mRNA (35), whereas a decrease was not observed with stable insulin receptor knockdown (36). Together, these findings suggest an important link between the prosurvival actions of insulin and Pdx1. We also provide evidence that Foxo1 exclusion from the nucleus may be involved in the antiapoptotic effects of insulin. Foxo1 is an established upstream regulator of Pdx1 (7). Although Akt is known to phosphorylate Foxo1, we did not find evidence of increased phospho-Foxo1 in insulin-treated cells, suggesting that insulin does not regulate Foxo1 translocation via Akt. Indeed, Akt-independent control of Foxo by insulin has been described in hepatocytes (37).

Although our results do not exclude the possibility that Akt may modulate insulin's effects on islet apoptosis, Akt does not appear to play an essential role in this regard. Although previous studies have shown that acute Akt phosphorylation coincides with many prosurvival stimuli, no sustained increase in Akt phosphorylation was observed with insulin treatment, and a significant component of antiapoptotic insulin signaling remained in primary islets isolated from mice expressing a dominant negative Akt mutant. We have shown that β-cell mass, even in the context of high fat feeding, is not altered in these mice. Instead, these mice exhibited a defect in insulin secretion (19), as was the case in β-cell-specific IGF1 receptor knockout mice (22, 23). We cannot rule out the possibility that a small fraction of total islet Akt activity is sufficient to mediate the effects of insulin on apoptosis, although we have shown that this kinase-dead Akt transgene inhibits Akt-mediated substrate phosphorylation in isolated islets by ≈80% (19), and it is known that β-cells comprise ≈80% of islet cells. A definitive analysis of the role of Akt in islet physiology awaits the combined tissue-specific knockout of all three Akt isoforms.

The implication of nonclassical insulin signaling in β-cell survival prompted a screen for novel insulin targets by using state-of-the-art proteomic technology. The major confirmed finding of these studies was that bridge-1 levels are stimulated by insulin. This fits with a recently proposed role for bridge-1 in the control of Pdx1-dependent insulin gene transcription (38). However, our finding that bridge-1 may be restricted to the cytosol suggests that the effects of this protein on gene transcription may be indirect, possibly through the binding of Pdx1 in the cytosol. It is worth noting that bridge-1 originally was cloned as PSMD9, a subunit of the cytosolic 20S proteosome complex, and that it therefore may play a role in Pdx1 degradation (39). Reminiscent of the "bell-shaped" dose–response of insulin detailed in the present study, the positive effects of bridge-1 on insulin mRNA levels were self-limiting, with deleterious effects at high doses (40). Because 5- to 10-fold transgenic overexpression of bridge-1 reduced Pdx1 levels, increased β-cell apoptosis and resulted in diabetes (40), one might speculate that some of the deleterious effects of high insulin may involve bridge-1.

The identification of protein spots altered by insulin provides interesting starting points for future studies. These included gene products linked to the suppression of oxidative stress and also to mitochondrial metabolism; both pathways can be targeted by insulin (41–43). The observation that metabolic genes are the primary targets of Pdx1 (30) suggests that insulin may act through Pdx1 to regulate these proteins. In addition, many of the protein spots decreased by insulin were linked to the cytoskeleton, shown in previous work to be regulated by insulin in other cell types (44). Interestingly, insulin decreased a spot identified as cathepsin D, a lysosomal protease that has been linked to apoptosis in other systems (45). However, we did not consistently observe changes in total pro- or cleaved cathepsin D levels by Western blot, leaving open the possibility of other posttranslational modifications.

There is mounting evidence that β-cell apoptosis plays a critical role in the pathogenesis of type 2 diabetes (8, 15, 46–48). If the protective effects of insulin are also lost at high concentrations *in vivo*, then the hyperinsulinemia commonly found in type 2 diabetics may have additional deleterious effects on β-cell apoptosis and glucose homeostasis. In addition, the short-term protective effects of glucose and other secretagogues also may depend on autocrine insulin signaling, given that gene array analysis of insulin receptor-deficient MIN6 cells showed that most of the genomic effects of glucose are mediated through the insulin receptor (36). The protective effect of short-term exposure to stimulatory glucose concentrations required PI3K, further suggesting a role for autocrine insulin signaling in β-cell survival (49). Taken together, these results are potentially important for our understanding of the pathogenesis of type 2 diabetes because they illuminate a link between insulin resistance and the β-cell apoptosis that may lead to a defect in insulin secretion from the endocrine pancreas. The recent demonstration that insulin signaling is defective in islets from human type 2 diabetics (11) reinforces the pathophysiological significance of this pathway.

Pancreatic β-cell apoptosis is recognized as a key event in type 1 diabetes (50). Thus, understanding of the role of autocrine insulin signaling also may be important in the context of type 1 diabetes, especially because variation in the insulin gene has been genetically linked to type 1 diabetes and confers up to 10% risk (51–53). Prosurvival components of the insulin-signaling pathway such as Pdx1 represent possible targets in efforts to reduce apoptosis during the progression of the disease (50). Furthermore, controlling apoptosis during organ procurement, islet isolation/culture, and engraftment is a potential key to the success in clinical human islet transplantation (54).

In summary, the present study has demonstrated that insulin, when administered at the appropriate dose, has a potent antiapoptotic effects in human islets. These effects are mediated via effects on Pdx1 and involve changes in the expression of unexpected proteins in the pancreatic islet. These results provide a basis for the development of novel approaches to enhancing β-cell survival.

PHYSIOLOGY

Materials and Methods

Primary Islet Cell Culture. Mouse islets were isolated by using collagenase and filtration as described in ref. 55, and human islets were provided by the Human Islet Isolation Core at Washington University in St. Louis or the Ike Barber Human Islet Transplantation Laboratory in Vancouver. Islets were cultured in (>2 ml per 10 islets) RPMI medium 1640 with 100 units/ml penicillin/100 μg/ml streptomycin/10% FCS, pH 7.4 with NaOH in 35 × 10 mm Nunc suspension dishes (Nalge, Rochester, NY) at 37°C and 5% CO_2 (14, 15). Solutions were treated with recombinant human insulin dissolved directly in solutions at ≤2,000 μM. For serum-withdrawal experiments, all islets were washed three times in serum-free media.

Immunofluorescence and Ca^{2+} Imaging. Immunofluorescence analysis of dispersed islet cells was performed essentially as described in refs. 8 and 15 by using a Zeiss 200M microscope (Carl Zeiss, Thornwood, NY) equipped with a ×100 objective. Images were analyzed/quantified by using SlideBook software (Intelligent Imaging Innovations, Boulder, CO). The polyclonal Pdx1 antibody was a gift from Christopher Wright (Vanderbilt University, Nashville, TN). Ca^{2+} imaging was conducted as described in refs. 14 and 15.

Measurement of Apoptosis by Using PCR-Enhanced DNA Ladder Analysis. The ability of insulin to modulate apoptosis was assayed in groups of 3–5 islets cultured in serum-free conditions for 5–7 days. Serum-free conditions are an established inducer of apoptosis in many cell types, including β-cells, and is most appropriate for testing the effects of insulin, because regular commercial serum supplements may contain insulin or related growth factors. We tested serum from six different companies and found insulin concentrations between 14 pM and 30 pM (i.e., 1–3 pM insulin would be expected in our culture media). An examination of the time course of serum withdrawal induced apoptosis demonstrated significant effects at 5 and 7 days (data not shown). Our method for PCR-enhanced DNA laddering has been described in detail in ref. 15.

Proteomics. Our methods for fluorescence-based proteomics are outlined in SI Fig. 8 and detailed in *SI Methods*. Briefly, whole lysates of human islets from the three culture conditions were labeled with specific Cy dyes and run together on the same 2D gel, which was imaged by using a Typhoon 9400 (Amersham Biosciences). Individual spots were quantified by using Decyder-DIA software (Amersham Biosciences) and were considered significantly different if they were >2 standard deviations outside this distribution. In our experiments, this cutoff corresponded to a ≈1.4-fold change in volume ratio. Significantly different spots were manually selected, using fold-change and resolution criteria, for sequencing by using either MALDI-TOF/TOF (Proteomics 4700; Applied Biosystems, Framingham, MA) or LC-MS/MS (Q-STAR XL; Applied Biosystems).

Western Blot. Western blots were performed according to standard methods. Briefly, human and mouse islets (cultured in groups of 40 in 50 ml of media) were washed twice with PBS before adding cell lysis buffer with protease inhibitor (Cell Signaling Technology, Beverly, MA). Total lysates were sonicated and protein concentrations were determined by using the BCA protein assay (Pierce, Rockford, IL). Protein lysates (15–30 μg) were subjected to PAGE, transferred to poly(vinylidene) fluoride membranes, which then were blocked with I-block solution (Tropix, Bedford, MA), washed, and probed with primary antibodies (listed in *SI Methods*). Immunodetection was performed with SuperSignal West Pico Chemiluminescent Substrate (Pierce).

Statistical Analysis. Statistical analyses were performed by using Student's unpaired *t* test or one-way ANOVA (followed by Fisher's PLSD post hoc test), where appropriate. Differences were considered significant when $P < 0.05$. Results are presented as mean ± SEM. All experiments were replicated on islets from more than three donors.

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